Apoptosis of the Human Neutrophils under a Lipopolisaccharide (LPS) of *Proteus mirabilis* Condition Observed in Real Time by Atomic Force Microscopy (AFM).

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ABSTRACT

Atomic force microscopy has been used for studying the process of apoptosis of the human neutrophils under a lipopolisaccharide in real time. In present paper it has established the increase of cells membranes rigid and the change of volume and height of neutrophils after addition of LPS *P. mirabilis 210*. Young's module was used for estimated the mechanical properties of neutrophils' membranes. Young's module of intact cells was 1.9 ± 0.3 kPa. It was increased significantly after addition of lipopolisaccharide.

Keywords: apoptosis, lipopolisaccharide, atomic force microscopy

1. INTRODUCTION

Apoptosis is a process of controlled cell removal in organism [1]. Lipopolysaccharide (LPS) is a powerful stimulator of many cells types and induces apoptosis in these cells. LPS has a clearly inflammatory effect and is also thought to induce apoptosis in several cell types, including neutrophils. Neutrophils exert key functions during the process of inflammation. However, they do not always play a positive role in the homeostasis of the immune system. Under some circumstances, neutrophils have deleterious effects [2]. Must of investigation have studied to activities the factors of inflammation (ex. cytokines) as agents which cause apoptosis [3]. However, to observe of change the cells' morphology during the deleterious effects of LPS is very important too. AFM is capable of real time imaging in vitro of living cells with high resolution [4].

The goal of present work was to receive the images of apoptosis process in dynamics after addition of *Proteus mirabilis 210* LPS and to measure the mechanical properties change of neutrophils' membranes.

2. MATERIALS AND METHODS

Human neutrophils were isolated from venous blood of healthy volunteers by centrifugation through ficoll-verografin density gradient (Pharmacia, Sweden) using densities of 1.077 and 1.116 g/ml. Erythrocytes were lysed with ice-cold isotonic NH4CL solution (155 mM NH4 CL, 10 mM KHCO3 , 0.1 mM EDTA, pH 7.2-7.4) for 12 min. The remaining granulocytes were washed with PBS and resuspended in HBSS at a concentration of $0.5 \cdot 10^6$ cells/ml.

LPS was isolated from bacterial cells [5]. The strains of *Proteus* were incubated (37°C, 18 h), washed with PBS, resuspended in 0.1? tris - ? ?1 (pH 8.05) and incubated (37°C, 12 h). After that they were centrifuged, resuspended, added 0.05 M EDTA and incubated (37°C, 10 min, in Thermomixer 5437 MERCK, Germany). 0.06? ? gCl_2 was added and centrifuged. A supernatant was refined and used as source of LPS.

Luminol-dependent chemiluminescence. Neutrophil suspension ($5 \cdot 10^5$ cells/ml.), containing luminol (10^{-5} M, Serwa, USA) was mixed with 0.1 ml the LPS of *Proteus.* Kinetic of LDCL has been registered during 60 min by chemiluminescence counter "Beta-1" (Medapparatura, Ukraine).

Atomic force microscopy (AFM). The cells morphology was investigated by SPM (Solver Bio ™ NT-MDT Co., Russia). We used contact V-shape SiN_4 probe (Veeco Co.) with spring constant 0.01 Nm, curvature radius of tip about 40 nm. Neutrophils were incubated in Petri's dishes with LPS.

3. RESULTS AND DISCUSSION

The result of neutrophils' activation by LPS of *Proteus* was measured during 60 min. Data (index of activation, which was calculated relatively control – spontaneous chemiluminescence without LPS) are shown in table 1.

So, we observed the significantly decrease of respiratory burst intensification on 15 min. after addition 0.1 ml LPS of *P.mirabilis 210* by luminol-dependent chemiluminescence. Suppression of neutrophils' respiratory activity was saved during of the observation time for cells (60 min). All other LPS (*P.mirabilis 120, P.vulgaris 296, P.vulgaris 856*) stimulated respiratory burst of neutrophils during 60 min of the observation.

The same result was received by AFM. LPS of all strain, which was investigated, did not cause modification of cells structures (during 60 min), except LPS of *P.mirabilis 210.* Change of cells morphological structure was exposed on 10 min after incubation with LPS of *P.mirabilis 210*. The volume of cells consecutively increased for some time after addition LPS and exceed initial volume of the cells in 1.8 – 2.1 times on 15 min (Fig.1, Tab.2).

Table 1

Luminol-dependent chemiluminescence of neutrophils, which was activated with LPS of *Proteus*

LPS	Index of activation	
	Index of peak/suppression chemiluminescence (I max)	Index of total sumlight (S)
P.mirabilis 120	4,09 ± 0,81*	3,03 ± 0,55*
P.mirabilis 210	0,61 ± 0,07*	0,65 ± 0,02*
P.vulgaris 296	2,98 ± 0,71*	2,28 ± 0,04*
P.vulgaris 856	2,39 ± 0,38*	1,99 ± 0,23*

* At the 0.05 level, the means are significantly different.

Table 2

Change of the neutrophils' parameters after addition LPS of *P.mirabilis 210* (on example of one cell)

	Int	**10 min**	**15 min**	**55 min**	**60 min**	**71 min**	**79 min**	**83 min**
Vol, mkm	146	246	248	242	238	184	240	202
Area, mkm	156	224	201	152	124	94	120	105
H max, nm	2128	2377	2589	2870	3374	3178	3538	3272

As you can see the volume and height of cells change constantly. They increase only to 40 min of the observation. Much more likely, a cell reconstructs internal structures under the action of apoptosis' factor at this time. However, the parameters of cells are very sloppy after 50 min.: cell now enlarges now reduces sizes. We suppose this is connected with forming and separation of the apoptosis bodies. We studied formation and separation of the apoptosis bodies after 50 min of the observation (Fig.2). Cells changed the morphological parameters (volume and height) during 3 h. Young's module was used for estimated the mechanical properties of neutrophils' membranes. Young's module of intact cells was 1.9 ± 0.3 kPa. After LPS addition Young's module has increased from 3 to 25 kPa.

So, in present paper it has established the increase of cells membranes rigid and the change of volume and height of neutrophils after addition of LPS *P. mirabilis 210*.

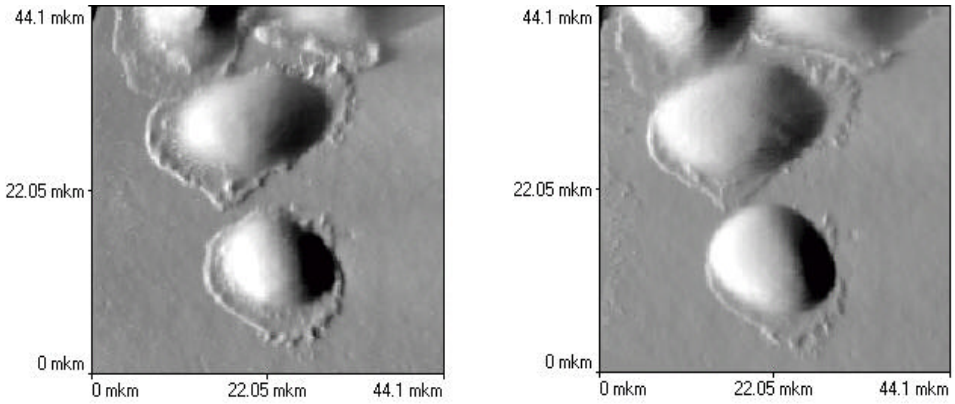

Fig.1. Human neutrophils a) before addition of LPS *P. mirabilis 210* (intact cell);
b) on 15 min after addition of LPS *P. mirabilis 210*.

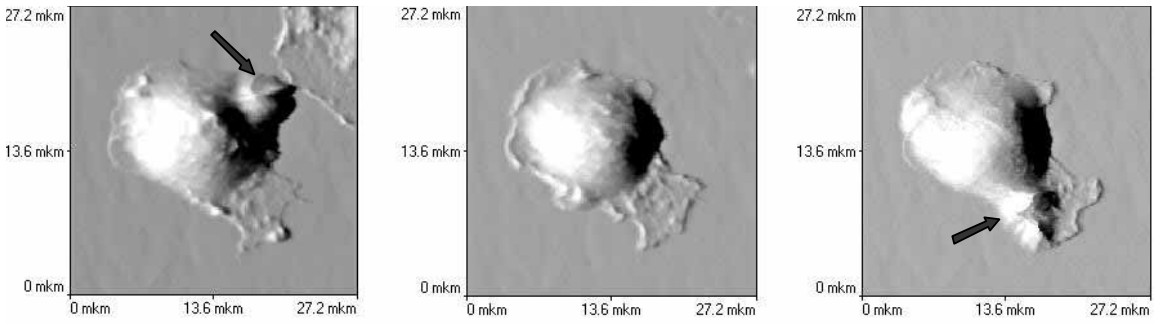

Fig.2. Human neutrophil after addition of LPS *P. mirabilis 210*
a) on 64 min;
b) on 71 min ;
c) on 83 min.
(Formation of the apoptosis bodies indicated by the arrows).

3. CONCLUSION

Results of investigation show that the Lipopolisaccharide of *Proteus mirabilis 210* produced apoptosis of the human neutrophils. As result of this process the cells swelled. After that they formed and separated the apoptosis bodies. The rigid of cells membranes was increased and we studied it using Young's module.

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